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Phylogenomic Analysis of the *PEBP* Gene Family from Kalanchoë

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Abstract: The *PEBP* family comprises proteins that function as key regulators of flowering time throughout the plant kingdom and they also regulate growth and plant architecture. Within the *PEBP* protein family, three subfamilies can be distinguished in angiosperms: MOTHER OF FT AND *TFL1*-like (MFT), FLOWERING LOCUS T-like (FT-like), and TERMINAL FLOWER1-like (*TFL1*-like). Taking advantage of the genome sequences available from *K. fedtschenkoi* and *K. laxiflora*, we performed computational analysis to identify the members of the *PEBP* gene family in these species. The analyses revealed the existence of 11 *PEBP* genes in *K. fedtschenkoi* and 18 in *K. laxiflora*, which are clustered in two clades: *FT*-like and *TFL1*-like. The *PEBP* genes had conserved gene structure and the proteins had highly conserved amino acid sequences in the positions crucial for the protein functions. The analysis of Ka/Ks ratio revealed that most recently duplicated genes are under positive selection. Despite being an economically important genus, the genetics underlying the regulation of flowering in Kalanchoë is poorly understood. The results of this study may provide a new insight into the molecular control of flowering that will allow further studies on flowering control in Kalanchoë.

Keywords: BROTHER OF FT AND *TFL1*; gene duplication; *Kalanchoë fedtschenkoi*; *Kalanchoë laxiflora*; FLOWERING LOCUS T; molecular phylogeny; TERMINAL FLOWER 1

1. Introduction

The family of phosphatidylethanolamine-binding proteins (*PEBP*s) is a group of proteins present in all eukaryote kingdoms. Despite extensive sequence conservation, *PEBP* proteins act as regulators of various signaling pathways to control growth and differentiation [1,2]. Phylogenetic studies suggest that the *PEBP* gene family can be divided into three subfamilies: *MOTHER OF FT AND TFL1*-like (*MFT*-like), *FLOWERING LOCUS T*-like (*FT*-like), and *TERMINAL FLOWER 1*-like (*TFL1*-like) [1,3]. It is thought that the *MFT*-like clade is the evolutionary ancestor to the *FT*-like and *TFL1*-like clades. A duplication of an ancestral *MFT*-like gene might have given rise to the *MFT*-like clade and the *FT*/*TFL1*-like clade. Further diversification of function and a second duplication resulted in the emergence of separate *FT*-like and *TFL1*-like clades [3,4]. A recent study revealed that the *MFT*-like subfamily exists in both basal land plants (bryophytes and pteridophytes) and seed plants (gymnosperms and angiosperms), while *FT*-like and *TFL1*-like genes are only found in gymnosperm and angiosperms. This suggests that the first duplication event took place after the divergence of basal land plants from the common ancestor of seed plants, while the second duplication event occurred before the divergence of seed plants. Additionally, within the three

subfamilies, more recent gene and/or genome duplications were observed in both angiosperms and gymnosperms further expanding the *PEBP* gene family [3].

The most studied functions of the *PEBP* gene family members concern the involvement of *FT* and *TFL1* homologs in controlling flowering time. Despite the high amino acid similarity FT and *TFL1* proteins have opposite activities: the FT protein can act as florigen [5] moving in the phloem from leaves to the shoot apex, while *TFL1* functions as a repressor in the shoot apex [6]. This antagonistic activity requires interaction with a common partner the bZIP transcription factor FD [7,8]. The complex of FD with FT/*TFL1* likely contains other proteins including 14-3-3 proteins that mediate these interactions [9].

Although in angiosperms the *FT*- and *TFL1*-like genes were previously thought to be primarily involved in the control of the transition to reproductive development, recent studies on perennial species suggest a more general role in controlling the growth and termination of meristems. The *TFL1* ancestor underwent two separate duplication events in the common ancestor of angiosperms, which created three lineages corresponding to *TFL1*, *BROTHER OF FT AND TFL1* (*BFT*), and the *CENTRORADIALIS* homolog (*ATC* or *CEN*) [10]. The *TFL1*/*BFT*/*CEN*-like genes control inflorescence meristem identity and delay transition to the reproductive phase [11] and are involved in growth and dormancy cycles [12], and seasonality of flowering in perennials [13,14]. Furthermore, the proteins from the *TFL1*/*CEN*/*BFT*-like subfamily can act as anti-florigen, a transmissible flowering repressor [15]. In many plant species, numerous *FT*-like genes arose from gene duplication events that might have led to subfunctionalization or neofunctionalization [16]. The *FT*-like genes were demonstrated to function in flower repression [17,18], vegetative growth [19], and storage organ formation [20,21]. Among angiosperms, *MFT*-like genes are thought to have a conserved function in regulation of seed germination via abscisic acid and gibberellic acid signaling pathways [22–25].

The Kalanchoë genus comprises ~140 species distributed on Madagascar, Southern and Eastern Africa, and to some extent, tropical Africa, the Arabian Peninsula, and Southern Asia. The species are mainly perennial succulent shrublets or shrubs, rarely small trees. However, they can also be perennial to biennial or rarely annual herbs [26]. Economically, the genus ranks as the second most important group of potted plants in Europe mainly due to high popularity of *Kalanchoë blossfeldiana* and its interspecific hybrids. The genus presents a wide range of attractive traits that can be of commercial value [26,27]. However, numerous species are difficult both, to induce flowering and control the time of flowering [28–30]. Generally, the flowering induction in Kalanchoë is determined by photoperiod. Within the genus two photoperiodic groups have been identified in respect to requirement for induction of flowering. They include short day (SD) plants, i.e., plants that are flower induced when exposed to a period of short days, and long-short day (LSD) plants, i.e., plants that require a dual sequence of photoperiods, which include species belonging to the former Bryophyllum genus sensu stricto [26]. There are, however, other factors, such as temperature and light intensity, that can greatly influence flower induction in Kalanchoë [31,32]. Moreover, some species have a long juvenile phase [33,34]. Apart from the significance of Kalanchoë as ornamental plants, the species of this genus have long been viewed as important models for the study of ecologically relevant modification of photosynthesis; the Crassulacean Acid Metabolism (CAM). *Kalanchoë fedtschenkoi* is now viewed as an emerging model system for functional genomics of CAM. Currently, genome sequences are available for *K. fedtschenkoi* and *K. laxiflora*, members of Bryophyllum section (*Kalanchoe fedtschenkoi* v1.1 and *Kalanchoe laxiflora* v1.1, DOE-JGI) [35] being the first sequenced species in the eudicot lineage Saxifragales. Interestingly, both induction of flowering and switch from C3 photosynthesis to CAM can be induced by photoperiod in Kalanchoë species; however, it is unknown if the output pathways of these processes are interconnected [36]. The members of the Kalanchoë genus include also several species with a broad range of ethnomedicinal uses. Therapeutic action of Kalanchoë plants is attributed to bufadienolides, a group of poly-hydroxy steroid hormones, displaying pharmacological activities such as anticancer, anti-inflammatory and cardioactive effects [37].

In this study, we performed computational analysis to identify members of the *PEBP* gene family from two Kalanchoë species taking advantage of available genome sequence data. We have

systematically analyzed the gene structure, gene family evolution and protein attributes to identify relevant targets for future functional genomic studies.

2. Materials and Methods

2.1. Identification of PEBP Sequences

The sequences were obtained by annotation and using full-length *A. thaliana* sequences as query sequences in BLASTP and TBLASTX searches against genomes and proteomes of *K. fedtschenkoi* Raym.-Hamet & H. Perrier and *K. laxiflora* Baker (Phytozome: *Kalanchoe fedtschenkoi* v1.1, *Kalanchoe laxiflora* v1.1, DOE-JGI). Additionally, recovered Kalanchoë protein sequences were used as query in BLASTP searches against Kalanchoë proteomes. The *A. thaliana* sequences were obtained from GenBank®: *AtFT* (NM_001334207.1), *AtTFL1* (NM_120465.3), *AtTSF* (NM_118156.2), *AtATC* (NM_128315.4), *AtBFT* (NM_125597.2), *AtMFT* (NM_101672.4) and proteins: AtFT (BAA77838.1), At*TFL1* (NP_196004.1), AtTSF (NP_193770.1), AtATC (NP_180324.1), AtBFT (NP_201010.1), and AtMFT (NP_173250.1). The Kalanchoë genes were named as *KfFT1* to *KfFT7*, *KfTFL1.1* to *KfTFL1.3*, and *KfBFT1* for *K. fedtschenkoi*, and *KlFT1* to *KlFT11*, *KlFTL1*, *KlTFL1.1* to *KlTFL1.4*, and *KlBFT1* to KlBFT2 for *K. laxiflora*, based on their accession IDs and database gene annotations (Table 1). Obtained protein sequences were analyzed for the presence of *PEBP* domains using the CDD database [38].

Table 1. Characterization of *PEBP* genes and proteins from Kalanchoë.

No.	Accession IDs	Gene Name	No. of Transcripts	Protein Length (aa)	Molecular Weight (kDa)	pI	Instability Index
1	Kaladp0007s0057	*KfFT1*	1	177	20.21	8.88	41.81; U [1]
2	Kaladp0031s0098	*KfFT2*	1	181	20.31	8.75	44.11; U
3	Kaladp0055s0469	*KfFT3*	1	178	20.21	9.09	38.93; S [2]
4	Kaladp0055s0470	*KfFT4*	1	176	19.88	7.72	38.51, S
5	Kaladp0060s0252	*KfFT5*	1	180	20.28	8.39	38.23; S
6	Kaladp0099s0141	*KfFT6*	1	181	20.41	7.73	51.05; U
7	Kaladp1016s0006	*KfFT7*	1	177	19.97	8.76	44.12; U
8	Kalax.0016s0040	*KlFT1*	1	180	20.27	8.39	38.23; S
9	Kalax.0052s0013	*KlFT2*	1	203	22.90	7.47	44.14; U
10	Kalax.0084s0028	*KlFT3*	1	177	20.21	8.88	41.81; U
11	Kalax.0100s0001	*KlFT4*	1	177	20.02	7.72	37.86; S
12	Kalax.0100s0002	*KlFT5*	1	177	19.97	9.27	41.29; U
13	Kalax.0114s0057	*KlFT6*	1	177	19.96	8.76	43.51; U
14	Kalax.0189s0069	*KlFT7*	1	177	20.25	8.88	42.24; U
15	Kalax.0399s0008	*KlFT8*	1	181	20.39	7.73	50.63; U
16	Kalax.0435s0026	*KlFT9*	1	181	20.41	7.73	51.05; U
17	Kalax.0644s0009	*KlFT10*	1	178	20.21	9.09	38.93; S
18	Kalax.0798s0001	*KlFT11*	1	176	19.88	7.72	38.51; S
19	Kalax.0508s0010	*KlFTL1*	1	112	12.28	5.24	32.41; S
20	Kaladp0011s0878	*KfTFL1.1*	1	179	20.39	8.35	33.13; S
21	Kaladp0093s0128	*KfTFL1.2*	1	175	19.89	8.73	54.72; U
22	Kaladp0095s0167	*KfTFL1.3*	1	173	19.39	7.96	46.78; U
23	Kalax.0010s0108	*KlTFL1.1*	2	179	20.36	8.35	27.75; U
				145	15.89	5.62	36.31; S
24	Kalax.0018s0096	*KlTFL1.2*	1	173	19.39	7.96	46.78; U
25	Kalax.0073s0028	*KlTFL1.3*	1	175	19.88	8.73	54.72; U
26	Kalax.0154s0031	*KlTFL1.4*	1	175	19.88	8.73	54.72; U
27	Kaladp0096s0031	*KfBFT1*	1	177	20.06	9.16	50.66; U
28	Kalax.0003s0185	*KlBFT1*	1	176	19.88	7.93	49.42; U
29	Kalax.0635s0001	*KlBFT2*	1	176	19.85	9.16	48.94; U

[1] U: unstable, [2] S: stable

2.2. Phylogenetic Analysis

Alignment of the *PEBP* domains from Kalanchoë *PEBP* genes was conducted using ClustalW. Multiple sequence alignment of *PEBP* proteins of Kalanchoë species, *Arabidopsis thaliana* (BAA77838.1, NP_196004.1, NP_201010.1, NP_173250.1, NP_193770.1, NP_180324.1), *Oryza sativa* (BAO02979.1, BAO03159.1, Q656A5, Q9ASJ1), *Chrysanthemum seticuspe* (BAL14659.1, BAN89465.1), *Fragaria vesca* (NP_001266951.1, AEP23097.1), *Malus domestica* (ADP69290.1, ACL98164.1, BAG31959.1, BAD06418.1, BAG31957.1, BAG31958.1), and Populus species (AFU08239.1, AFU08240.1, Q6TXM3, B9HPZ6, Q2PPJ2, B9ID58) was performed using Clustal Omega. MEGA 7.0 software was used to build the neighbor-joining phylogenetic tree from the protein and gene sequence alignment using the following parameters; p-distance model, pairwise gap deletion, and 1000 bootstraps. DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST) analysis [39] was performed to determine the homologous relationships among *PEBP* proteins from Kalanchoë and other species.

2.3. Protein Characterization

The protein analysis was performed using ProtParam [40] for prediction of protein length, molecular weight, pI and instability index.

To identify conserved motifs in *PEBP* family proteins, the online MEME v.4.12.0 (Multiple Expectation Maximization for Motif Elicitation) [41] program was used with minimum motif length of 6 and maximum of 100, the maximum number of motifs was set to 20. Protein structure prediction was performed using SWISSMODEL and Swiss PdbViewer [42].

2.4. Gene Structure Analysis

The information about the gene length and distribution of exons and introns in *PEBP* genes was obtained from Phytozome. The intron/exon organization for *PEBP* genes was determined by aligning the CDS sequences to their corresponding genomic DNA sequences and using the result as the input for graphical display at the Gene Structure Display Server v2 [43].

Gene CDS sequences were aligned and percent identity matrix was generated using Clustal Omega with default parameters [44].

2.5. Gene Duplication Analysis

MEGA 7.0 software [45] was used to calculate rate of nonsynonymous substitutions (Ka) and synonymous substitutions (Ks). Codon-based testing of purifying selection for analysis between sequences was conducted using the modified Nei-Gojobori (assumed transition/transversion bias = 2) method. All ambiguous positions were removed for each sequence pair. The dates of the duplication events (T—Duplication time; Mya—Million years ago) between the duplicated Kalanchoë genes associated with terminal branches in the tree species clades were calculated by the equation [46]

$$T = Ks/2\lambda \times 10^{-6} \text{ Mya, the } \lambda = 1.5 \times 10^{-8} \quad (1)$$

Diversity analysis (nonsynonymous substitutions per nonsynonymous site; Ka/synonymous substitutions per synonymous site—Ks) was performed using the DnaSP v5.10 program [47] with a sliding window mode (window size 50, step 10).

3. Results

3.1. Identification of PEBP Sequences

BLAST searches using *PEBP*-like nucleotide and protein sequences from *A. thaliana* against plant protein and nucleotide databases of two Kalanchoë species resulted in 29 *PEBP*-like genes being retrieved; predicted to encode 30 proteins. There were 11 genes in *K. fedtschenkoi* that is a diploid species and 18 in *K. laxiflora* that is a tetraploid species. Based on annotations provided by the Phytozome database the genes and corresponding proteins were assigned to three groups: *FT*-like

(seven genes from *K. fedtschenkoi* and 12 from *K. laxiflora*), *TFL1*-like (three genes from *K. fedtschenkoi* and four from *K. laxiflora*), and *BFT*-like (one gene from *K. fedtschenkoi* and two from *K. laxiflora*). The detailed information about Phytozome gene IDs assigned gene names and number of transcripts are included in Table 1.

3.2. Comparative Phylogenetic Analysis

The unrooted neighbor-joining phylogenetic tree was constructed using predicted amino acid protein sequences from Kalanchoë and six other species in which functional assessment of FT and *TFL1* functions was confirmed by transgenic approach [4,15,48] (Figure 1).

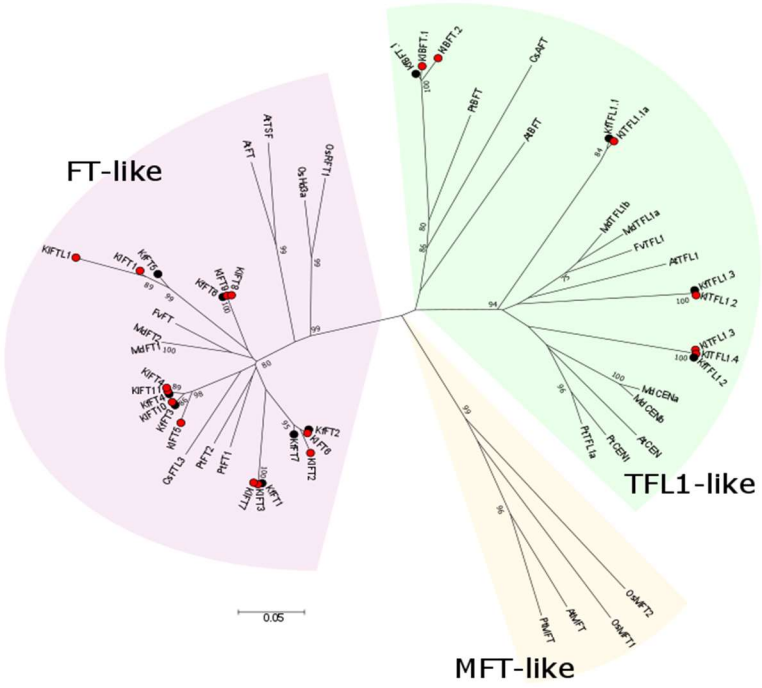

Figure 1. Phylogenetic analysis of proteins from the *PEBP* family. Full-length amino acid sequences of *PEBP* family proteins from Kalanchoë. *Arabidopsis thaliana*—At (BAA77838.1, NP_196004.1, NP_201010.1. NP_173250.1, NP_193770.1, NP_180324.1); *Oryza sativa*—Os (BAO02979.1, BAO03159.1, Q656A5, Q9ASJ1); *Chrysanthemum seticuspe*—Cs (BAL14659.1, BAN89465.1), *Fragaria vesca*—Fv (NP_001266951.1, AEP23097.1); *Malus domestica*—Md (ADP69290.1, ACL98164.1, BAG31959.1, BAD06418.1, BAG31957.1., BAG31958.1); and Populus species—Pt (AFU08239.1, AFU08240.1, Q6TXM3, B9HPZ6, Q2PPJ2, B9ID58) were used to generate an unrooted neighbor-joining radial tree constructed with 1000 bootstrap replications (values <70 are not displayed). The background colors were used to highlight the groups of FT-like, *TFL1*/ BFT-like, and MFT-like proteins. Different colors were used to distinguish between proteins of *Kalanchoë fedtschenkoi* and *Kalanchoë laxiflora*.

The phylogenetic analysis revealed that tree main clades MFT-like, FT-like, and *TFL1*/CEN/BFT-like could be distinguished in the constructed tree as described previously [2]. In the FT-like clade, proteins of *K. fedtschenkoi* and *K. laxiflora* grouped very closely together forming three distinct groups. Furthermore, the Kalanchoë FT-like proteins shared high homology with those from other perennial plants. Within *TFL1*/CEN/BFT-like clade, the BFT-like proteins formed clearly separated subclade containing Kalanchoë BFT-like proteins together with Populus and *Arabidopsis* BFT, as well as CsAFT, an antiflorigen protein from Chrysanthemum. The *TFL1*-like proteins from Kalanchoë grouped together with both *TFL1*- and CEN-like proteins.

The comparative phylogenetic analysis confirmed that *PEBP*-like proteins from Kalanchoë are homologous to FT-like, *TFL1*-like and CEN-like, and BFT-like proteins from other species, and that MFT-like sequences could not be identified in Kalanchoë.

The gene homology analysis using DELTA-BLAST further confirmed that Kalanchoë *FT*-like, *TFL1*-like, and *BFT*-like genes are homologous to the *A. thaliana FT* gene (69–78% sequence similarity), the CEN/TFL genes (62–77% sequence similarity), and the *BFT* gene (66–67% sequence similarity), respectively (Table S1). Furthermore, the homology analysis comparing the Kalanchoë *PEBP* genes and sequences available in the Genbank for various species confirmed that Kalanchoë sequences have high homology with perennial species (Table S2).

3.3. Kalanchoë Protein Characterization

The length of the proteins ranged from 112 to 203 aa (average: 176 aa; median: 177 aa) in FT-like proteins and 145 to 179 aa (average: 172 aa; median: 175 aa) in *TFL1*-like proteins. The BFT-like proteins were either 176 or 177 aa. All identified proteins were characterized by a conserved *PEBP* domain. Domain analysis of primary transcript results from the CDD database confirmed the presence of the *PEBP* domain in the N-terminal regions of these proteins, except for KlFTL1 that contained only a partial *PEBP* domain. The multiple alignment of domain sequences followed by phylogenetic analysis confirmed the presence of three protein groups corresponding to FT-like, *TFL1*-like, and BFT-like proteins (Figure 2).

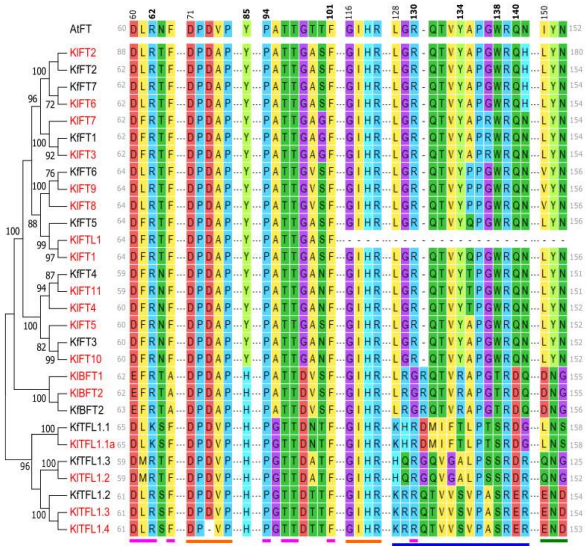

Figure 2. Amino acid alignment of deduced *PEBP* family protein sequences from Kalanchoë and *Arabidopsis thaliana* FT proteins. The colors indicate amino acids of different biochemical properties as obtained through MEGA 7.0. The sequences were aligned using ClustalW. Alignment of amino acid sequences of *PEBP* proteins at the 14-3-3 interaction interface is underlined in pink. The conserved DPDXP (Asp-Pro-Asp-X-Pro) and GIHR (Gly-Ile-His-Arg) motifs that compose anion-binding sites are underlined in orange. The conserved segment B (positions 128–141, exon 4) is underlined in blue and segment C (149–151, exon 4—XYN triad) is underlined in green. Amino acid positions indicated on the top of the sequence alignment are based on the *Arabidopsis thaliana* FT protein sequence BAA77838.1. Bold amino acid positions distinguish residues that are considered crucial for *PEBP* flowering inductive and repressive functions and 14-3-3 protein binding according to references [4,7,8,9,49]. In the present alignment arginine at position 62 (R62), proline at position 94 (P94), phenylalanine at position 101 (F101), and arginine at position 140 (R130) are predicted to participate in binding between *PEBP* proteins and 14-3-3 proteins. Tyrosine at position 85 (Y85) is present in all FT-like proteins, while histidine at position 85 (H85) is present in *TFL1*-like proteins. FT-like proteins contain tyrosine at position 134 (Y134), while *TFL1*-like proteins contain non-tyrosine amino acids. All FT-like proteins contain tryptophan at position 138 (W138), while *TFL1*-like contain non-tryptophan amino acids. At position 140, FT-like proteins contain glutamine (Q140), while *TFL1*-like proteins contain aspartic acid (D140) or glutamic acid (E140).

The Kalanchoë *PEBP* proteins shared 40% to 100% identity at the amino acid sequence level (Table S3). The FT-like protein identity ranged between 81% to 96% in *K. fedtschenkoi* and 71% to 99%

in *K. laxiflora*. There was between 72% to 100% sequence identity among FT-like proteins between both species. The *TFL1*-like protein identity ranged between 65% to 69% in *K. fedtschenkoi* and from 50% to 100% in *K. laxiflora*. There was between 51% to 100% sequence identity among *TFL1*-like proteins between both species. The BFT-like proteins in *K. laxiflora* showed 98% identity, while between species the identity was 98%.

The *PEBP* proteins had highly conserved amino acid sequences in the positions crucial for the protein functions (description in Figure 2). The differences in the conserved residues were observed in Kf*TFL1*.1 and Kl*TFL1*.1a at position 62 where lysine (K) was identified instead of arginine (R) that has, however, similar properties, and a deletion of the second aspartic acid (D) in the DPDXP motif that forms the anion-binding site was observed in the Kl*TFL1*.4 protein. In addition, in BFT-like proteins the arginine residues were observed at positions 129 and 131, but not at position 130.

The molecular weights of the predicted molecules were ~19–23 kDa for all proteins except from KlFTL1 (12.28 kDa) and Kl*TFL1*.1b (15.89 kDa). The pIs were 5.24 and 9.27 and instability indexes were between 27.75 and 54.72. Of 30 analyzed proteins, 10 were predicted as stable and 20 as unstable. The detailed information about the proteins' molecular weight, isoelectric points and instability indexes are included in Table 1.

The MEME protein motif search tool identified a total of ten conserved motifs in Kalanchoë *PEBP* proteins ranging from 6 to 44 amino acids. The motif pattern appeared to be highly conserved among the proteins (Figure S1).

The protein models of KfFT3 and Kf*TFL1*.3 were constructed based on the similarity with crystal structure of *A. thaliana* FT, 1wkpA, and *TFL1*, 1wkoA (Figure 3). KfFT3 shared 76% identity with 1wkpA, and *TFL1* shared 69% identity with 1wkoA.

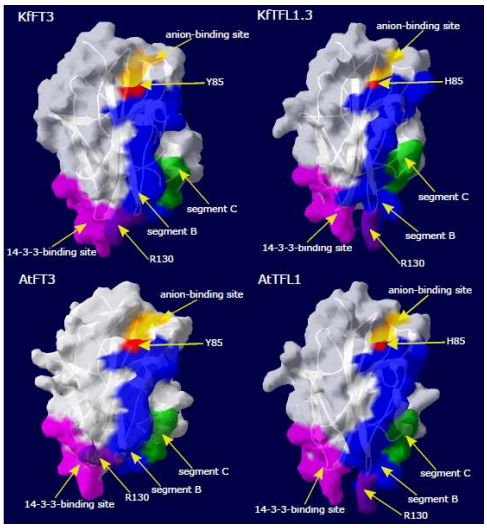

Figure 3. Predicted structure of KfFT3 and Kf*TFL1*.3 in comparison to AtFT (*A. thaliana* 1wkpA) and At*TFL1* (*A. thaliana* 1wkoA) proteins [8]. The external loop segment B is represented in blue, segment C (XYN triad) in green, putative 14-3-3-binding site in pink, and anion binding site in orange color. The conserved Y85/H85 residue crucial for FT/*TFL1* antagonistic activities are shown in red. The conserved R130 residue of segment B important for 14-3-3 binding activity is represented in violet.

3.4. Gene Structure

The length of the coding regions of *PEBP*-like genes in Kalanchoë ranged from 339 to 612 bp, with an average of 526 bp (*FT*-like: 531 bp; *TFL1*-like: 518 bp; *BFT*-like: 532 bp). All the genes of Kalanchoë were predicted to encode one transcript, except *KlFTL1.1*, that had two transcripts (Figure 4). Analysis of intron–exon distribution revealed that both *FT*-like and *TFL1*-like genes conserved the characteristic genomic organization for the gene family, with four exons and three introns, except *KlFTL1* and *KlFTL1.1b* (secondary transcript of the gene). The length of the first exon in *FT*-like was between 198 and 285 bp (average: 211 bp, median: 207). The second and third exons were highly

conserved with the length of 41 and 62 bp, respectively, except from *KlFTL1*, which had third exon of 64 bp. The fourth exon in *FT*-like group was between 224 and 236 bp (average: 228 bp and median: 230). The *TFL1*-like genes had a first exon between 198 and 216 bp (average: 207 bp, median 204 bp). Similarly, to *FT*-like genes, the second and third exons were highly conserved with 62 and 41 bp, respectively, except the *KlFTL1.1* gene (*KlFTL1.1b*), which had a third exon of 160 bp. The fourth exon in *TFL1*-like group was 221 bp in all the genes. All three *BFT*-like genes were characterized by structures comprising three exons and two introns. The first exon was 207 or 210 bp, while second and third exons were 103 and 221 bp, respectively, in all the genes.

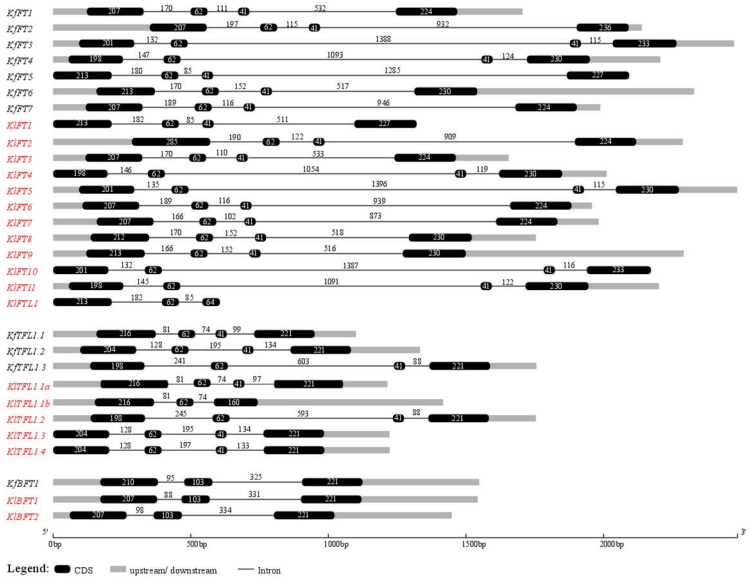

Figure 4. Structural organization of the *PEBP* family genes in Kalanchoë. The white boxes represent 5′ and 3′ untranslated regions (UTRs) and black boxes represent coding regions. The introns in coding region are presented as black lines. The scale bar at the base represents the length of the genes in bp. Information was retrieved from Phytozome: *Kalanchoe fedtschenkoi* v1.1, *Kalanchoe laxiflora* v1.1, DOE-JGI.

The introns had variable length in all the gene groups from 74 to 1396 bp. The 4-exon *FT*-like genes were characterized by a structure with two short introns (<200 bp) and one long intron (>500 bp). The long intron was found either in the second (12 out 18 genes) or the third position (six out 18 genes). The 4-exon *TFL1*-like genes had either all three short introns (<200 bp; five out of seven genes), or had a first medium length intron (~240 bp) and a second long intron (~600 bp) (two out of seven genes). The *BFT*-like genes were characterized by a first short intron (<100 bp) and one medium length intron (~320 bp).

The identity of coding sequences for all *PEBP*-like genes in Kalanchoë ranged between 50.5% and 100% (Table S3). The *FT*-like gene identity ranged between 72% to 99 % in *K. fedtschenkoi* and 69% to 99% in *K. laxiflora*. There was between 69% to 100% sequence identity among *FT*-like genes between both species. The *TFL1*-like gene identity ranged between 65% to 68% in *K. fedtschenkoi* and 56% to 99% in *K. laxiflora*. There was between 56% to 100% sequence identity among *TFL1*-like genes between both species. The *BFT*-like genes in *K. laxiflora* showed 98% identity, while between species the identity was 97% and 99%.

3.5. Gene Duplication Analysis

The number of synonymous and nonsynonymous substitutions per site of the duplicated *PEBP* genes in Kalanchoë associated with terminal branches in the tree species were determined using MEGA 7.0. We determined that average GC content in the third codon position that was 59% (Table S5). Thus, Ks values were used for calculation of the time of duplication events. The gene duplication events in *K. fedtschenkoi* in *FT*-like clade included one recent event dated approximately 0.9 MYA,

while other occurred between 5.5 and 16.0 MYA. The duplication events in *TFL1/BFT*-like clade might have occurred earlier between 20.5 and 25.4 MYA. In *K. laxiflora* the gene duplication events might have occurred more recently. The recent duplication events in FT-like and *TFL1/BFT*-like clades occurred approximately 0.9–3.2 MYA, with only one older duplication event dated approximately 20.7 MYA. The recent duplications in *K. laxiflora* can be associated with whole genome duplication and might have resulted in divergence and formation of a new species. The duplication events are presented in the species gene trees (Table 2 and Figure 5).

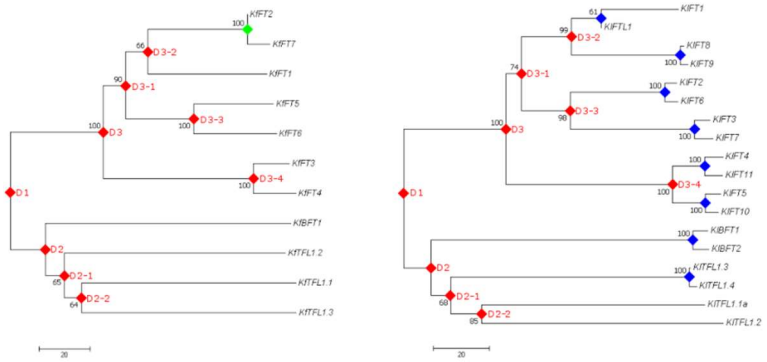

Figure 5. Species *PEBP* gene family trees in *Kalanchoë fedtschenkoi* (**left**) and *Kalanchoë laxiflora* (**right**) with proposed gene duplication events; red marks indicate probable respective gene duplication events (D1 to D3-4) that occurred before divergence of *K. fedtschenkoi* and *K. laxiflora*; blue marks indicate gene duplications observed only in *K. laxiflora* associated with probable WGD that lead to speciation; and the green mark indicates gene duplication in *K. fedtschenkoi* that occurred after split between *K. fedtschenkoi* and *K. laxiflora*.

Codon-based test of purifying selection revealed that the majority of duplicated gene pairs were under purifying selection, except one *FT*-like gene pair in *K. fedtschenkoi* (*KfFT2–KfFT7*) and three FT-like gene pairs in *K. laxiflora* (*KlFT1–KfFTL1*, *KlFT2–KfFT6* and *KlFT5–KfFT10*) (Table 2).

Table 2. The Ks values and estimated absolute dates for the duplication events between the duplicated Kalanchoë genes associated with terminal branches in the tree species clades.

Gene 1	Gene 2	CDS Identity	Ks [1]	Mya [2]	Purifying Selection [3]
KfFT1	*KfFT2*	83.2	0.461	15.4	yes
KfFT1	*KfFT7*	82.5	0.480	16.0	yes
KfFT2	*KfFT7*	99.0	0.027	0.9	no
KfFT3	*KfFT4*	94.2	0.166	5.5	yes
KfFT5	*KfFT6*	88.2	0.222	7.4	yes
KfTFL1.1	*KfTFL1.2*	65.3	0.727	24.2	yes
KfTFL1.1	*KfTFL1.3*	67.6	0.650	21.7	yes
KfTFL1.2	*KfTFL1.3*	66.7	0.763	25.4	yes
KfTFL1.1	*KfBFT1*	64.8	0.614	20.5	yes
KfTFL1.2	*KfBFT1*	62.1	0.811	27.0	yes
KfTFL1.3	*KfBFT1*	62.6	0.644	21.5	yes
KlFT1[5]	*KlFTL1*	95.0	0.063	2.1	no
KlFT2	*KlFT6*	98.3	0.053	1.8	no
KlFT3	*KlFT7*	97.8	0.095	3.2	yes
KlFT4	*KlFT11*	97.6	0.053	1.8	yes
KlFT5	*KlFT10*	98.1	0.026	0.9	no
KlFT8	*KlFT9*	99.3	0.026	0.9	yes
KlBFT1	*KlBFT2*	97.9	0.096	3.2	yes
KlTFL1.1a	*KlTFL1.2*	68.2	0.621	20.7	yes
KlTFL1.3	*KlTFL1.4*	99.4	0.041	1.4	yes

[1] Ks—Rate of synonymous substitutions; [2] Mya—Estimated dates of duplication events (million years ago); [3] results of the codon-based test of purifying.

To further evaluate the sequence diversity in *FT* and *TFL1* homologs, we performed a sliding window analysis of rates of pairwise nonsynonymous (Ka) and synonymous (Ks) substitutions (Figure 6). While exon 4 of the FT proteins had almost no amino acid substitutions indicating strong purifying selection, exon 4 of *TFL1* had higher Ka/Ks ratio, suggesting more relaxed selection.

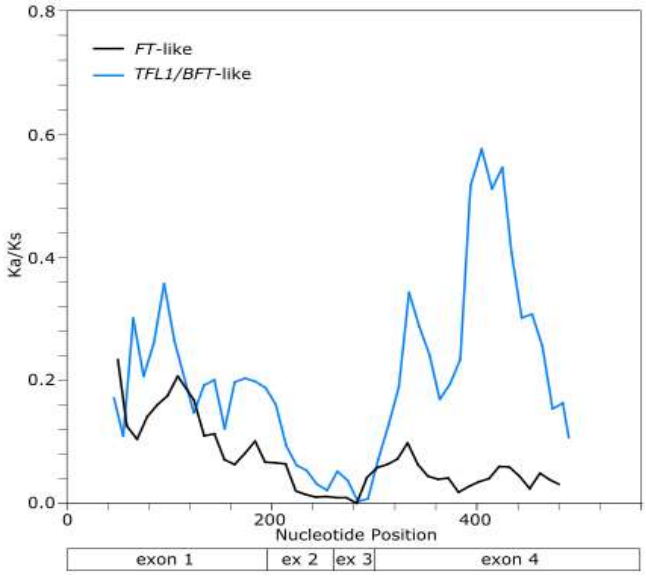

Figure 6. Average pairwise ratio of nonsynonymous (Ka) to synonymous substitutions (Ks) (sliding window, window size 50, step 10) of Kalanchoë *PEBP* genes. *TFL1/BFT* genes show an excess of nonsynonymous substitutions in exon 4.

4. Discussion.

The *PEBP* family is one of the most ancient gene families, with a highly conserved gene structure and high protein sequence similarities across species [1,2]. Its members include genes with very important functions in flowering induction and plant architecture [3,4]. The *PEBP* genes, particularly *FT*-like and *TFL1*-like genes, have been identified and their detailed functions were studied in many plant species including model plants, crop and vegetable species, and ornamental plants [7,9,15,21,50,51]. In this study, we focused on *PEBP* genes from Kalanchoë that is an important genus of flowering ornamental plants. So far, there is no information available about *PEBP* genes in Kalanchoë or other species of the Crassulaceae family. Therefore, using available genome sequence data we identified *PEBP* gene members in two species—*K. fedtschenkoi* and *K. laxiflora*—and characterized their gene structures, gene family evolution, and protein features.

The *FT* gene is expressed in the phloem companion cells of the leaves under flower inductive conditions and corresponds to an FT protein of approximately 20 kDa with a theoretical isoelectric point of ~8–9 [52–55]. The FT protein is a key component of the florigen complex, which is translocated from leaves to SAM where it promotes flowering [4,6,56]. The *TFL1* gene is expressed in the SAM [7] and the *TFL1* protein is a signal that is translocated from the inner SAM cells to the outer cells and coordinates the cell identity [57]. With regard to flowering, the FT protein promotes flowering in plants, while [6] *TFL1* represses flowering [49]. However, other *TFL1*-like proteins can be expressed in the vascular tissues and translocate to SAM to repress flowering [15,49,58,59].

Previous functional studies have shown that the *PEBP* family can be divided into three major functional clades [2,3,24]. In the present study, phylogenetic analysis of the deduced protein sequences demonstrated that Kalanchoë proteins can be classified into an FT-like clade and a *TFL1*/BFT-like clade. The FT-like proteins were closely related to proteins of perennial species, which were reported to regulate induction of flowering [19,48,60–62]. Thus, these proteins are likely the FT homologs that can regulate flower transition and initiation in Kalanchoë. Similar to FT-like proteins, the *TFL1*-like proteins showed close relation to *TFL1*-like proteins that controls inflorescence

meristem identity and delays the transition to the reproductive phase at the SAM [63–65]. Interestingly, the BFT-like proteins showed high homology to a mobile floral inhibitor from *Chrysanthemum seticuspe* [15]. Old physiological studies suggested an existence of a flowering inhibitor produced in leaves of Kalanchoë plants [66,67]. Thus, BFT-like proteins might fulfill the function of a mobile flower repressor. In angiosperms, all three *PEBP* gene families were identified in all species (including *Phoenix dactylifera* which was previously reported to lack *MFT*-like genes) [3]. However, in our study we were unable to identify *MFT*-like sequences in neither *K. fedtschenkoi* nor *K. laxiflora*. The inability to identify *MFT*-like genes might be due to genome misassembly resulting in mosaic gene sequences or gene loss in the assembly due to collapse of the repetitive surroundings [68,69]. However, even though very unlikely, the loss of *MFT*-like genes cannot be ruled out. *K. fedtschenkoi* and *K. laxiflora* represent the only species with available genome data from the Saxifragales order. Thus, the comparison with other closely related species is currently not possible.

It has been demonstrated in *A. thaliana* that FT and *TFL1* may have an interchangeable roles by replacing a single amino acid [7] or protein segment [8,16] (Figure 2). Differences in FT/*TFL1*-like protein activities might be due to their binding affinity towards FD and/or 14-3-3 proteins. In the Kalanchoë *PEBP* protein alignment, we identified four amino acids, i.e., R62, P94, F101, and R130, predicted to participate in binding of 14-3-3 proteins [9,21]. Amino acid alignment revealed that Kalanchoë FT-like proteins had conserved tyrosine at position 85 (Y85) characteristic for floral promoters, while *TFL1*-like proteins had conserved histidine at position 85 (H85) characteristic for floral repressors [7]. Generally, FT-like proteins contain a region called segment B, which forms a loop in the protein structure and is essential for FT-like proteins to function as floral promoters. FT-like proteins usually contain tyrosine at position 134 (Y134) and tryptophan at position 138 (W138) in segment B, whereas the flowering repressor proteins contain non-tyrosine and non-tryptophan amino acids in these positions, respectively [4,8,49]. Additionally, FT-like proteins contain a triad region—Segment C—that is required for full functionality of FT-like proteins but not *TFL1*-like proteins [4,8]. Therefore, the presence of the mentioned residues indicates that Kalanchoë FT-like can act as flower activators and *TFL1*/BFT-like as flower repressors.

The number of *PEBP*-like genes found in different plant species varies greatly with up to 19 genes found in *Glycine max* and 24 copies identified in *Musa acuminate* [3]. The average number of *PEBP* genes in monocots (~17) was shown to be roughly twice the number in dicots (~8) [3]. In our study, we identified 11 genes in *K. fedtschenkoi* and 18 in *K. laxiflora.* These numbers are similar to eudicot species, such as *Brassica rapa* (12 *PEBP* genes), *Solanum lycopersicum* (12 *PEBP* genes), and *G. max* (19 *PEBP* genes), which were demonstrated to experience additional whole genome duplication events throughout their evolutionary history [3]. Recent analysis of the *K. fedtschenkoi* genome provided strong evidence for two ancestral WGD events in this species [35]. The comparison between *PEBP* trees from *K. fedtschenkoi* and *K. laxiflora* suggests that the latter species experienced a recent WGD event that based on Ks values associated with lateral branches took place between 0.9 and 3.2 MYA (Table 2 and Figure 5). Thus, the number of *PEBP* genes is consistent with diploid/ tetraploid nature of the analyzed Kalanchoë species.

The investigated *PEBP* genes had highly similar sequences and exon-intron structures (Table S3 and Figure 4). Particularly, the exonic structures were highly conserved with characteristic for *PEBP* gene family exon 2 (62 nt) and exon 3 (41 nt) invariable in size in *FT*-like and *TFL1*-like genes. The only exception from *FT*-like genes includes *KlFTL1* that appears to be a pseudogene lacking the entire 4th exon. However, incomplete gene sequence can be also a result of a mistake during genome assembly. Furthermore, the *BFT*-like genes from both species demonstrated novel gene structure with three exons and two introns resulting from a fusion between exon 2 and exon 3 (103 nt). Even though *PEBP* genes have highly conserved gene structures, some species exhibit novel features such as additional intron/exon in *Musa acuminate* [3] and *Chenopodium rubrum* [70], and exon fusion in *Zea mays* of *FT*-like genes [24]. *KlTFL1.1* was predicted to have alternative transcript as a result of downstream alternative usage of transcription start site (TSS). Alternative usage of TSSs and alternative splicing are key mechanisms to generate gene variation in eukaryotes. Both mechanisms are known to play important roles in tissue-specific gene expression and functional variation, which

have significant impact on biological processes [71]. Alternative splicing in *TFL1*/*CEN* paralogs in saffron was reported to influence terminal flowering and flowering time [72]. Thus alternative transcript of the *TFL1.1* gene in *K. laxiflora* may be relevant in spatiotemporal expression of the gene. However, it is also possible that downstream TSS might produce a truncated protein whose function is deteriorated or lost.

The evaluation of sequence diversity in *FT* and *TFL1* homologs revealed that the majority of the most recently duplicated genes are under positive selection (Table 2). Furthermore, a more detailed sliding window analysis of Ka and Ks revealed strong differences in the substitution rates in exon 4 between *FT*- and *TFL1*-like genes. This is consistent with previous studies showing that segment B situated in exon 4 evolved very rapidly in *TFL1* orthologs, but is almost invariant in *FT* orthologs. Thus, the residues encoded by the fourth exon of *FT* determine the function of the protein [1,8].

In Kalanchoë, flowering time is an important economic trait. Despite efforts to understand the mechanisms underlying the impact of photoperiod and temperature on the induction of flowering, little is known about the genetic basis of flower transition. The FT protein is a key integrator among different flowering pathways in angiosperms that promotes flowering [73]. In many plant species *FT* homologs regulate aspects of plant development in response to photoperiod and temperature [48,51,62,74,75]. Thus, the extended family of *FT* genes identified in Kalanchoë might be of significant relevance to flower induction in the response to different environmental cues. In breeding programs and commercial cultivation of many perennial plants a prolonged juvenility is one of the major problems. Modifying the FT/*TFL1* ratio can change the flowering time [73]. Furthermore, *TFL1* expression may be relevant for prevention of precocious flowering [76]. The overexpression of an *FT* homolog in poplar induced early flowering [62] and downregulation of a *TFL1* homolog accelerated the flowering age [64]. The continuous flowering trait in roses and strawberries is associated with loss of function of the *TFL1* homolog. The continuous flowering plants are characterized by short juvenile phase and rapid flowering after seed germination [77]. Thus, modification of FT/*TFL1* expression may provide possibility to shorten the juvenile phase in Kalanchoë species and obtain plants that flower for a long period of time with no need of environmental control.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Alignments of the amino acid sequences of the *PEBP* proteins, Figure S2: Results of MEME motif analysis with *PEBP* proteins from Kalanchoë, Table S1: Results of homology analysis of Kalanchoë genes with *Arabidopsis thaliana*, Table S2: Results of homology analysis of Kalanchoë genes with various species, Table S3: CDS and protein identity in Kalanchoë, Table S4: Predicted subcellular localization of Kalanchoë *PEBP* proteins, Table S5: GC content (%) at first (P1), second (P2) and third (P3) codon position in *PEBP* family genes in Kalanchoë.

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